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Nonsteroid Nuclear Receptors: What Are Genetic Studies Telling Us about Their Role in Real Life?

Review

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Introduction

Many of the cloned vertebrate nuclear receptors have been extensively studied in vitro in transfected cultured cells. Unfortunately, this approach is far from being physiological (e.g., the amount of receptor may greatly exceed the normal cellular amount) and does not necessarily reflect what is occurring in vivo. Ligand "manipulation" may provide some clues about functions of the cognate receptor. However, the actual involvement of a particular receptor in the known functions of its ligand needs to be demonstrated in animals in which the functions of the receptor have been abrogated by genetic means. This is particularly true in cases when several receptors bind the same ligand or when the ligand also interacts with other proteins (e.g., cellular retinoic acid–binding proteins [CRABPs] in the case of retinoic acid [RA]). Genetic studies are even more crucial for assigning functions to orphan receptors. Targeted disruption (knockout) via homologous recombination has recently been used to generate mice lacking receptors (Table 1). Expression of dominant negative receptors or antisense mRNA has also been employed. Together with some nuclear receptor mutations associated with pathological conditions, knockouts have led to significant advances in our knowledge of the physiological functions of several nuclear receptors, but have also raised unexpected problems. These studies and their implications are summarized and discussed here except those concerning steroid hormone receptors (see Beato et al., 1995 [this issue of *Cell*]).

Orphan Receptors

Function of FTZ-F1

The FTZ-F1 gene protein (also known as SF1 or Ad4BP) binds to promoter regions of several steroidogenic enzymes (for references see Sadovsky et al., 1995) and of the anti-Müllerian hormone (AMH; Shen et al., 1994). Comparison of the expression pattern of FTZ-F1 and AMH in the embryonic gonad strongly suggests that FTZ-F1 could be a key regulator of AMH expression, and thus control the differentiation of the male genital apparatus (Shen et al., 1994).

FTZ-F1 null mutants lack gonads and adrenals and die during the first days of life, probably owing to the absence of adrenal function since they can be rescued by corticosteroids administration (Luo et al., 1994, 1995a; Sadovsky et al., 1995; Shinoda et al., 1995). These mice also lack the ventromedial hypothalamic nucleus (Ikeda et al., 1995; Shinoda et al., 1995). In addition, gonadotropic markers (luteinizing hormone, follicle-stimulating hormone, and the gonadotropin-releasing hormone [GnRH] receptor) could not be detected in the pituitary (Ingraham et al., 1994), but were induced by GnRH treatment (Ikeda et al., 1995). As GnRH hypothalamic neurons are present in FTZ-F1 mutants, the abnormal pattern of gene expression in gonadotropic cells probably reflects an impaired GnRH release, which might be caused by an abnormal or absence of interaction between the ventromedial hypothalamic nucleus and the GnRH neurons (Ikeda et al., 1995). Thus, FTZ-F1 appears to be critical for the development, function, or both of the hypothalamic–pituitary–gonadal axis. Ironically, the null mutants cannot be used to assess FTZ-F1 function in either the control of steroidogenic enzyme or AMH expression, as tissues in which these regulations take place are not formed.

Function of NGFI-B/NURR77

Nerve growth factor–induced receptor NGFI-B is an immediate-early protein whose expression is induced by a variety of stimuli. NGFI-B may be involved in the control of the hypothalamic–pituitary–adrenocortical axis, as NGFI-B transcripts are strongly induced by stress in the adrenal cortex and in the paraventricular hypothalamic nucleus (for references see Crawford et al., 1995). Together with FTZ-F1, NGFI-B has also been implicated in the control of steroidogenic enzyme expression in adrenal glands (for references see Crawford et al., 1995). NGFI-B might also act in the control of activation-induced apoptosis of thymocytes and T cell hybridomas, since NGFI-B is rapidly and strongly induced after activation of immature thymocytes and since expression of a "dominant negative" NGFI-B or NGFI-B antisense transcripts prevents activation-induced apoptosis of T cells (Liu et al., 1994; Woronicz et al., 1994). It was therefore a surprise that NGFI-B null mutants display no detectable defect, with respect to both T cell apoptosis and function of the hypothalamic–pituitary–adrenocortical axis (Lee et al., 1995a; Crawford et al., 1995). The apparent discrepancy between the normal phenotype of the NGFI-B null mice and the functions postulated above for NGFI-B most probably reflects functional redundancy (compensation) by closely related receptors (NURR1 and NGFI-Bγ; see Crawford et al., 1995). The absence of an apoptosis phenotype in NGFI-B mutant thymocytes indicates that the dominant negative and antisense approaches lack specificity and are prone to unexpected artefacts, as their effects cannot be attributed to the loss of function of NGFI-B only.

Function of DAX1

DAX1 is an unusual member of the nuclear receptor family, since it contains a nuclear receptor-like ligand-binding domain (LBD), but an unrelated DNA-binding domain (DBD). Loss-of-function mutations of DAX1 have been identified as the genetic basis of X-linked adrenal hypoplasia congenita and X-linked hypogonadotropic hypogonadism, characterized by structural abnormalities of the adrenal glands and gonads, resulting in impaired steroidogenesis

Table 1. Viability of Nuclear Receptor Knockout Mice

Mutated Receptor(s)	Lethality in Utero	Perinatal Lethality	Reduced Postnatal Viability	Normal Viability	References
Orphans					
FTZ-F1			+		Luo et al., 1994; Sadovsky et al., 1995; Shinoda et al., 1995
NGFI-B				+	Lee et al., 1995a
HNF4	+				Chen et al., 1994
COUP-TF-I		+			Qiu et al., 1995
COUP-TF-II	+				Qiu et al., 1995
PPARα				+	Lee et al., 1995b
TRs					
TRβ				+	Forrest et al., 1995
RXRs					
RXRα	+				Sucov et al., 1994; Kastner et al., 1994
RXRβ	+[a]	+[a]		+[a]	Kastner et al., 1996
RARs					
RARα		+[a]	+[a]	+[a]	Lufkin et al., 1993
RARβ				+	Luo et al., 1995b
RARγ		+[a]	+[a]	+[a]	Lohnes et al., 1993
RARα1				+	Lufkin et al., 1993; Li et al., 1993
RARβ2				+	Mendelsohn et al., 1994b
RARγ2				+	Lohnes et al., 1993
RAR double mutants					
RARα/γ	+[a]	+[a]			Lohnes et al., 1994
RARα/γ		+			Lohnes et al., 1994
RARα/β2		+			Lohnes et al., 1994
RARα1/β2		+			Lohnes et al., 1994
RARβ2/γ		+			Lohnes et al., 1994

[a] This phenotype is only partially penetrant.

(Zanaria et al., 1994; Muscatelli et al., 1994). It is intriguing that the DAX1 and FTZ-F1 mutations affect the same tissues, raising the question whether these receptors may belong to the same regulatory pathway. Interestingly, a putative FTZ-F1 response element has been found in the *Dax1* promoter (Burris et al., 1995).

Function of HNF4

Hepatocyte nuclear factor 4 (HNF4) binds to response elements present in several liver-specific genes (for references see Chen et al., 1994). Together with its restricted expression in liver, intestine, and kidney, this suggests an important role for HNF4 in the mediation of tissue-specific gene expression in these organs. HNF4 mutants die at approximately 8.5 days postcoitum (dpc) (Chen et al., 1994) and exhibit extensive cell death in the ectoderm at 6.5 dpc. Gastrulation and mesoderm formation appear delayed by 24 hr and are greatly impaired. The underlying mechanisms are unknown, but the primary defect is likely to reside in the visceral endoderm, which selectively expresses HNF4 at early stages. Chen et al. (1994) have suggested that ectodermal cell death and subsequent morphogenetic defects may reflect the impaired production by the visceral endoderm of factors necessary for survival of ectodermal cells. Owing to this early embryonic lethality, the HNF4 mutation failed to produce an in vivo model for the study of HNF4 function in liver-specific gene expression.

Function of COUP-TFs

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) (COUP-TF-I and COUP-TF-II, also known as Arp-1; see Qiu et al., 1995) are highly conserved in evolution, as their DBD and putative LBD amino acid sequences are almost identical to those of their Drosophila homolog, Seven-up. This strong evolutionary pressure suggests that COUP-TFs probably perform important functions. Both COUP-TF-I and COUP-TF-II have been mutated in the mouse, and preliminary results indicate that both genes are essential, since COUP-TF-I mutants die perinatally, and COUP-TF-II mutants die in utero (see Qiu et al., 1995). Thus, despite extensive overlap in their patterns of expression and a high sequence similarity, COUP-TFs perform some important nonredundant functions.

Function of PPARs

In transfected cells, peroxisome proliferator–activated receptors α, β, and γ (PPARα, PPARβ, and PPARγ) are activated by a variety of fatty acids and several synthetic compounds that induce peroxisome proliferation in the liver and hepatocarcinogenesis (reviewed by Green and Wahli, 1994). As heterodimers with retinoid X receptors (RXRs), PPARs have been broadly implicated in the control of lipid metabolism, since putative PPAR response elements have been characterized in the promoter regions

of several genes encoding enzymes involved in fatty acid catabolism (β- and ω-oxidation). In addition, the PPARγ2 isoform was shown to induce adipocitic differentiation of preadipocyte cell lines (Tontonoz et al., 1995).

So far, only PPARα null mutants have been reported (Lee et al., 1995b). They are phenotypically normal, but appear refractory to the action of peroxisome proliferators. Hepatomegaly, peroxisome proliferation, and induction of enzymes involved in fatty acid catabolism (e.g., acetylcoenzyme A oxydase, bifunctional enzyme) were not observed. Thus, PPARα mediates the response to peroxisome proliferators. Although these observations support the idea that PPARα is involved in the control of lipid catabolism, the "normal" physiological function of PPARα remains uncertain, since the basal level of expression of most of the above enzymes was not affected in the mutants. However, even though it was not emphasized by Lee et al. (1995b), the basal expression of bifunctional enzyme appeared to be markedly reduced. It will therefore be interesting to investigate whether β-oxydation is impaired in these animals.

Function of Thyroid Hormone Receptors

Studies of thyroid hormone–deficient animals and human nutritional iodine deficiency have demonstrated a key role for thyroid hormones in development of the central nervous system, postnatal maturation of the myocardium, and pituitary–thyroid axis homeostasis. There are two thyroid hormone receptor (TR) genes, TRα and TRβ. Mutations in the TRβ gene have consistently been found in patients with the dominantly inherited general resistance to thyroid hormone (GRTH) syndrome, characterized by high circulating levels of thyroid hormones and thyroid-stimulating hormone and by symptoms similar to those arising from thyroid hormone deficiency (e.g., hearing defects, mental retardation, learning disabilities, and emotional disturbance). These mutations, which always correspond to point mutations located in the LBD, decrease or abolish ligand binding, but not heterodimerisation with RXR nor DNA binding (Collingwood et al., 1994, and references therein). Mutant receptors act as dominant negatives and inhibit T_3-dependent activation by wild-type TR in vitro. GRTH patients exhibit several other defects, notably various congenital skeletal malformations, that cannot be explained on the basis of thyroid hormone deprivation (Refetoff, 1982). Thus, a dominant negative TRβ might also interfere with events not normally mediated by TRs, possibly via RXR titration. In contrast, no TRα mutation has been found in GRTH patients. Thus, the absence of TRα may lead only to subtle defects, or, alternatively, a mutation in the TRα gene may be lethal.

TRβ null mutant mice display the syndrome of pituitary resistance to thyroid hormone and impaired hearing (Forrest et al., 1995). Interestingly, deafness has been associated with various human thyroid disorders.

Function of the Vitamin D Receptor

No knockout of the vitamin D receptor (VDR) gene has been reported yet, but VDR mutations are associated with hypocalcaemic vitamin D–resistant rickets in humans. Hypocalcaemic vitamin D–resistant rickets is a recessive disease characterized by high levels of 1,25-dihydroxyvitamin D_3, severe rickets, hypocalcaemia, secondary hyperparathyroidism, and total absence of hair in severe cases. Homozygous point mutations in the VDR gene have been identified in several kindreds (Hughes et al., 1988; Kristjansson et al., 1993; Wiese et al., 1993). These mutations fall into two major categories: mutations in the DBD, abrogating DNA binding, and nonsense mutations resulting in loss of ligand binding. It is unclear why dominant negative mutations similar to those of TRβ have not been found for VDR. One possibility would be that TR, but not VDR, dominant negative alleles are overexpressed relative to wild-type alleles.

A strong association has been found between apparently functional VDR allelic variants, a decreased bone mineral density, and the rate of bone mineral density change in relation to calcium intake (Morrison et al., 1994). How these allelic variants affect bone mineralization is unknown, but there are nucleotide changes in their 3′ untranslated region that appear to be associated with a lower mRNA stability (Morrison et al., 1994).

Function of Retinoid Receptors

Until recently, the physiological functions of retinoids were mainly inferred from studies on vitamin A–deficient (VAD) animals and, rather paradoxically, from the defects caused by administration of pharmacological doses of RA (reviewed by Means and Gudas, 1995). VAD studies have shown that vitamin A (retinol) is required during pre- and postnatal development and in adult life. After birth, retinol is indispensable for survival, growth, reproduction, and vision and also for the maintenance of numerous tissues. Widespread squamous metaplasia of various epithelia and degeneration of seminiferous tubules and photoreceptors are hallmarks of the postnatal VAD syndrome, and RA can prevent or reverse the effects of a postnatal VAD diet, with the exception of night blindness and photoreceptor degeneration (for references see Chambon, 1994). Interestingly, conceptuses of VAD dams exhibit a large number of congenital malformations (i.e., the fetal VAD syndrome; Figure 1; for references see Lohnes et al., 1994; Mendelsohn et al., 1994a).

Two families of retinoid nuclear receptors have been characterized (Leid et al., 1992). Retinoic acid receptors (RARs) (types α, β, and γ and their isoforms α1, α2, β1 to β4, and γ1 and γ2) are activated by all-*trans* and 9-*cis* RA, whereas RXRs (types α, β, and γ and their isoforms) are activated by 9-*cis* RA only. The complexity of retinoid signaling is further increased by the fact that, at least in vitro, RARs bind to their cognate response elements as heterodimers with RXRs. Moreover, RXRs, which can also bind in vitro to certain DNA elements as homodimers, are heterodimeric partners for a number of nuclear receptors, such as TRs, VDR, PPARs, and NGFI-B (reviewed by Mangelsdorf and Evans, 1995 [this issue of *Cell*]).

Null mutations of RARα, RARβ, or RARγ, as well as isoform-specific knockouts of RARα1, RARβ2/β4, and RARγ2, have been generated (Tables 1 and 2). Mice lacking RARβ, RARα1, RARβ2, or RARγ2 appear normal. In

Abnormalities of the fetal VAD syndrome	genotype of RAR mutants exhibiting similar congenital abnormalities
Respiratory tract defects	
• Agenic or hypoplastic left lung*	α/β2 #
• Hypoplastic right lung*	α/β2 #
• Lack of oesophago-tracheal septum*	α1/β2 → α/β2 #
Thinner "spongy" myocardium	α/γ (mild)
Heart outflow tract and aortic arch derivative abnormalities	
• Persistent truncus arteriosus (NCC)	α1/β2 → α/β2 #, α/γ #
• Dextroposed aorta (NCC)	α1/β2, α1/α2$^{+/-}$/γ, α/γ
• High ventricular septal defect*	α1/β2 → α/β2 #, α1/α2$^{+/-}$/γ → α/γ #
• Abnormalities of great arteries derived from aortic arches (NCC)	α1/β2 #, α/β2 #, α1/α2$^{+/-}$/γ → α/γ #
Diaphragmatic hernia *	α/β2
Ureter abnormalities	
• Agenesis*	α1/β2, α/β2,α/γ #
• Ectopia*	α1/β2, α/β2
Genital tract abnormalities	
- **Female** • Agenesis of the the oviduct and uterus *	
- Total	α/β2 #
- Partial	α1/β2 #, α/γ #
• Agenesis of cranial vagina*	α1/β2 → α/β2 #, α/γ #
- **Male** • Agenesis or dysplasia of vas deferens*	α1/α2$^{+/-}$/γ → α/γ #
• Agenesis of seminal vesicles*	α/γ #
Ocular abnormalities	
• Coloboma of the retina*	α/γ #
• Coloboma of the optic nerve*	β2/γ #, α/γ #
• Retrolenticular membrane* (NCC)	αβ2 #, α/γ #
• Unfused eyelids* (NCC)	α1/α2$^{+/-}$/γ → α/γ #
• Small conjonctival sac* (NCC)	β2/γ #, α/γ #
• Abnormal corneal stroma (NCC)	β2/γ #, α/γ
• Absence of anterior chamber* (NCC)	β2/γ #, α/γ #
• Abnormal lens fibers	α/γ
• Agenesis of cornea, conjunctiva and eyelids* (NCC)	α/γ
• Shortening of ventral retina and ventral rotation of the lens	-
• Pre-natal retinal dysplasia	-

Figure 1. Almost All the Abnormalities of the Fetal VAD Syndrome Are Found in One or Several Combinations of RARα (Total or α1), RARβ2, or RARγ Double Mutants

Note that most of the abnormalities found in the α/γ mutants are absent in α1/γ and α1/α2$^{+/-}$/γ mutants. Asterisks indicate abnormalities that correspond to ontogenetic arrests. The abbreviation NCC indicates abnormalities attributed to faulty migration, differentiation, proliferation, or apoptosis of NCCs. A number sign indicates an abnormality that is completely penetrant. The arrow denotes an increase from partial to full penetrance of the defect. For further details see Lohnes et al. (1994) and Mendelsohn et al. (1994a).

contrast, RARα or RARγ null mutants (i.e., all α or γ isoforms disrupted) display some of the defects of the postnatal VAD syndrome that can be cured or prevented by RA administration, including poor viability, growth deficiency, and male sterility. These mutants also exhibit some congenital malformations (Table 2), which, however, are confined to a small subset of the tissues expressing these receptors. RAR double null mutants have been generated to test for a possible functional redundancy in the RAR family (Table 1; Figures 1 and 2). In contrast with RAR single mutants, most of the double mutants exhibit a dramatically reduced viability (Table 1). Furthermore, almost

Table 2. Postnatal Manifestations of RAR and RXR Single Knockouts

Genotype	Abnormalities	References
RARα1	None	Li et al., 1993; Lufkin et al., 1993
RARα	Decreased viability, growth deficiency, male sterility (degeneration of seminiferous epithelium); congenital malformations: webbed digits, homeotic transformations, and malformations of cervical vertebrae[a]	Lufkin et al., 1993; Lohnes et al., 1994
RARγ2	None	Lohnes et al., 1993
RARγ	Decreased viability, growth deficiency, male sterility (squamous metaplasia of seminal vesicle and prostate gland epithelia); congenital malformations: webbed digits, homeotic transformations, and malformations of cervical vertebrae, fusion of trachea rings, agenesis of Harderian glands	Lohnes et al., 1993
RARβ2	None reported	Mendelsohn et al., 1994b
RARβ	None reported	Luo et al., 1995b
RXRα$^{+/-}$	Growth deficiency	Kastner et al., 1994
RXRβ	Male sterility (defective spermiogenesis)	Kastner et al., 1996

[a] Low penetrance and expressivity.

Congenital Abnormalities not associated with the fetal VAD syndrome	RAR mutant genotype
Exencephaly*	α/γ
Skeletal abnormalities	
Agenesis or malformations of cranio-facial skeletal elements (NCC)	α1/α2$^{+/-}$/γ # (mild), α/γ #
Homeotic transformations and malformations of cervical vertebrae	α, γ → α1/γ #, α/γ #, α/β2 #, β2/γ
Limb bone agenesis and malformations	α/γ #
Appearance of atavistic skeletal structures	
• Pila antotica (NCC)	α1/γ #, α/γ #
• Pterygoquadrate cartilage (NCC)	α1/α2$^{+/-}$/γ → α/γ #, α1/β2 → α/β2, β2/γ
Ocular abnormalities	
Corneal-lenticular stalk*	α1/α2$^{+/-}$/γ, α/γ
Lens agenesis*	α/γ
Post natal retinal dysplasia	β2/γ2
Glandular abnormalities	
Harderian, sublingual, submandibular, glandular agenesis and dysplasia	γ, α1/γ, α/γ, β2/γ
Thymus, thyroid, parathyroid gland hypoplasia and ectopia (NCC)	α1/β2, α/β2, α1/α2$^{+/-}$/γ, α/γ
Other abnormalities	
Webbed digits	α, γ, β2/γ$^{+/-}$, α$^{+/-}$/β2
Abnormal cartilages (thyroid, arytenoid, cricoid, bronchial and/or tracheal rings)	γ, α1/γ, α/γ #, α/β2, β2/γ
Kidney agenesis or hypoplasia*	α1/β2, α/β2 #, α/γ #
Anal canal agenesis	αβ2

Figure 2. Congenital Malformations Absent in the Fetal VAD Syndrome Are Exhibited by RARα, RARβ2, or RARγ Single and Double Mutants

Asterisks indicate abnormalities that correspond to ontogenetic arrests. The abbreviation NCC indicates abnormalities attributed to faulty migration, differentiation, proliferation, or apoptosis of NCCs. A number sign indicates an abnormality that is completely penetrant. The arrow denotes an increase from partial to full penetrance of the defect. For further details see Lohnes et al. (1994) and Mendelsohn et al. (1994a).

all of the fetal VAD syndrome malformations are recapitulated in the different RAR double mutants (Figure 1). These findings demonstrate that RA is the active vitamin A derivative during development and that the effects of vitamin A are mediated by RARs. RAR double mutant fetuses also exhibit numerous abnormalities not found in VAD fetuses (Figure 2), which most probably reflects the difficulty to achieve, by dietary deprivation, a state of severe VAD compatible with pregnancy. Interestingly, in contrast with this crucial role of RARs in transducing the RA signal, CRABPI and CRABPII are not critically involved in these processes, as CRABPI/CRABPII double null mutant mice are essentially normal (Lampron et al., 1995).

RARs and the Neural Crest

Malformations of most of the structures derived from cranial and cardiac mesectoderm (mesenchymal neural crest) were observed in RAR double mutants (Figures 1 and 2; Lohnes et al., 1994; Mendelsohn et al., 1994a), notably in RARα/γ mutant mice. However, the first pharyngeal arch skeletal elements, which are derived from caudal midbrain and rostral hindbrain levels (i.e., rhombomeres 1 and 2) were intact or only mildly affected (Lohnes et al., 1994). Interestingly, the first arch neural crest cells (NCCs) appear to be embodied with a ground state morphogenetic program, which is also present in the second pharyngeal arch, where it is respecified by expression of *Hoxa-2* (Mark et al., 1995, and references therein). Frontonasal mesectodermal cells are also embodied with a similar program (Noden, 1983). Thus, the realization of at least part of the first arch ground state program does not require RA, whereas its modification both in the frontonasal and second arch mesectoderm involves RA-dependent processes. Since NCCs appeared with the emergence of vertebrates (Gans and Northcutt, 1983), RARs may have evolved to fulfil functions necessary for the development of mesenchymal NCC-derived structures. In this respect, it will be interesting to characterize the RAR that may be present in prochordates (e.g., amphioxus), since NCC are lacking in these immediate ancestors to vertebrates. Similarly, it is noteworthy that atavistic reptilian mesectodermal structures are generated in RAR mutant mice, namely, a pterygoquadrate cartilage (reptilian upper jaw cartilage) and a pila antotica (a skeletal element that, in reptiles, separates the trigeminal ganglion from the brain). These findings suggest that the RA signal has been used during evolution to modify ancestral developmental programs in the mesectoderm (see Lohnes et al., 1994; Mark et al., 1995).

RARs and Axial Patterning

RARγ and (to a much lesser extent) RARα single mutants show homeotic transformations and malformations of vertebrae, thus firmly establishing that RA plays an important role in the patterning of the main body anteroposterior axis (Lohnes et al., 1993, 1994). The penetrance and expressivity of these defects increase in a graded manner with subsequent loss of RARα1 and RARα2 alleles from the

RARγ null background (Figure 2; Lohnes et al., 1994). RARβ2 also appears to play a role in axial patterning, since RARα/β2 (but not RARα) mutants display a high frequency of anterior transformations of the sixth and seventh vertebrae (Lohnes et al., 1994). These transformations, which correspond to almost exclusively anteriorisations, are restricted to the cervical region and probably arise through altered expression of some 3′ *Hox* genes: some of them are similar to certain *Hox* loss-of-function transformations (Lohnes et al., 1994, and references therein); RA response elements that are functional in vivo are present in the promoter region of some *Hox* genes (Studer et al., 1994, and references therein; Frasch et al., 1995, and references therein); *Hox* gene expression was shown to be controlled by RA in embryonal carcinoma (EC) cells (Mavilio, 1993, and references therein). Why only cervical vertebrae are transformed in RAR single and double mutants is unclear. It may indicate that only the expression of some 3′ *Hox* paralogs, which specify the identities of anterior vertebrae, is critically controlled by RA, as suggested by the preferential RA induction of 3′ *Hox* genes in EC cells (Mavilio, 1993). It is also unclear why the main body axial patterning defects present in RAR double mutants are milder than expected from RA excess experiments (for further discussion of these points, see Conlon, 1995). The generation of RARα/β/γ triple mutants may further define the role of retinoid receptors in patterning of the main body anteroposterior axis.

RARs and Limb Morphogenesis

A role for RARs in limb formation, patterning, and growth has been suggested by teratogenic experiments, as systemic administration of excess RA can induce extra limb formation (Rutledge et al., 1994), and topical application of RA can mimic the effect of the zone of polarizing activity (ZPA) on anteroposterior limb patterning (reviewed by Tickle and Eichele, 1994). RARα/γ mutants display limb malformations, demonstrating, at the least, that RA is indispensable for limb morphogenesis. The majority of the forelimb defects in RARα/γ mutants involve the loss of anterior skeletal elements. This phenotype can be interpreted as loss of one of the functions of the wild-type ZPA, which is to promote the proliferation of limb bud mesenchyme (Lohnes et al., 1994, and references therein; Conlon, 1995, and references therein). However, RAR mutant limbs all show a clear anteroposterior asymmetry, suggesting that the "polarizing" activity of their ZPA is essentially preserved. Since RARβ is normally present in a region of the limb bud that overlaps with the ZPA, the possibility remains that inactivation of all three RARs might result in more dramatic effects on limb patterning and might even affect limb formation.

Role of RARs in Eye Development

Warkany and Schraffenberger (1946) reported half a century ago that the developing eye is the most sensitive organ to vitamin A deprivation. The spectrum of VAD-induced ocular malformations is almost fully recapitulated in RAR double null mutants (Figure 1). Thus, RA is clearly the vitamin A derivative required for eye morphogenesis, and RARs mediate its effects. These studies also demonstrate that distinct RARs are required at several steps of eye morphogenesis as well as in retinal histogenesis (Figures 1 and 2). Interestingly, in the absence of RARs some ocular structures undergo aberrant differentiation (i.e., the corneal epithelium keratinizes in RARα/γ mutants, whereas the primary vitreous body chondrifies in RARβ2/γ mutants; Lohnes et al., 1994).

Role of RARs in Reproduction

It has been claimed that retinol plays a unique role in spermatogenesis, as VAD-induced testis degeneration could not be reversed by RA administration (Thompson et al., 1964). The degeneration of the seminiferous epithelium in RARα null mutants matches closely that resulting from a VAD diet (Lufkin et al., 1993), indicating that RA, and not retinol, is the active retinoid required for spermatogenesis. The retinol requirement likely results from the existence of a blood–testis barrier preventing RA delivery to the adluminal compartment of the seminiferous tubules (for references see Lufkin et al., 1993). The sterility of RARγ mutants males, which is caused by a squamous metaplasia of the glandular epithelia of the prostate and seminal vesicle, also corresponds to a classical manifestation of the postnatal VAD syndrome (Lohnes et al., 1993). Thus, the retinoid signal, which is necessary for spermatogenesis and for the maintenance of the differentiated state of the epithelia of the male accessory glands, appears to be transduced by RARα and RARγ, respectively. The genital ducts are also severely affected in RAR compound mutant fetuses, as the oviducts, uterus, and cranial vagina are never formed in RARα/β2 mutants, and the vas deferens and seminal vesicles are agenic or severely malformed in RARα/γ mutants (Mendelsohn et al., 1994a).

Function of RARs in Skin

Retinoids are used to treat a variety of skin diseases, and RA can modulate epidermal morphogenesis in vitro (Darmon, 1991), suggesting that RA is important for skin formation and maintenance. Expression of a dominant negative RAR in basal keratinocytes has resulted in an inhibition of epidermal differentiation, with absence of formation of the spinous and horny layers, and in persistent expression of keratin 5 and 14 in suprabasal cells (Saitou et al., 1995). Furthermore, expression of a dominant negative RAR in suprabasal keratinocytes resulted in the absence of the lipid lamellar structure underlying the cornified envelope, with a concomitent loss of barrier function (Imakado et al., 1995). In marked contrast, RAR mutant mice have so far failed to reveal dramatic changes in the stratified morphology of the epidermis. The discrepancy between the dominant negative and the knockout results may reflect the presence of a low level of RARβ in RARα/γ double mutants or the interference of dominant negative RARs with other signaling pathways (e.g., TR, VDR, and PPAR through titration of RXRs; for further discussion see Andersen and Rosenfeld, 1995). Further studies are required to clarify the possible function of RARs in skin.

Function of RXRs

RXRs are thought to exert multiple functions in several signaling systems (reviewed by Mangelsdorf and Evans,

1995). Thus, the detection of an abnormal phenotype for an RXR mutant raises the question as to which of these pathways has been affected. Furthermore, despite the wealth of data dealing with the in vitro function of RXRs, it is essentially unknown which biological processes, if any, are specifically regulated by 9-*cis* RA-liganded RXRs and to which extent the heterodimeric partnerships characterized in vitro operate in vivo. To date, null mutations for RXRα and RXRβ have been engineered.

Function of RXRα

RXRα null mutants display a thin ventricular wall and die between 10.5 dpc and 17.5 dpc from cardiac failure (Sucov et al., 1994; Kastner et al., 1994; Dyson et al., 1995). A similar "spongy myocardium" phenotype has been described in VAD fetuses, suggesting that this ventricular defect reflects a role for RXRα in transducing a retinoid signal. However, as a milder form of spongy myocardium can occur in some RARα/γ double mutants (Mendelsohn et al., 1994a), the RXRα function in ventricular wall development may involve a heterodimeric partnership with RARs (see below). Early differentiated features (i.e., the presence of striated myofibrils) and a reduced rate of cell proliferation were observed in the RXRα$^{-/-}$ subepicardial ventricular myocytes as early as 8.5 dpc. Thus, RXRα prevents precocious differentiation of ventricular myocytes.

RXRα null mutants also display ocular abnormalities, notably malformations of the anterior segment of the eye and shortening of the ventral retina (Kastner et al., 1994). The function of RXRα in the morphogenesis of the anterior segment is likely to depend on a partnership with RAR3, as similar defects occur in RARα/γ and RARβ2/γ double mutants (Figure 1), and a strong synergy for malformation of these structures was observed when RXRα and RARγ mutations were combined (see below). In contrast, shortening of the ventral retina is one of the few abnormalities of the fetal VAD syndrome that has not been found in RAR mutants, raising the possiblity that the function of retinoids in ventral retina development could be mediated by RXRα, independently of RARs. Interestingly, mutation of *ultraspiracle* (*usp*), the Drosophila RXR homolog, results in a shorter ventral retina (Oro et al., 1992), which may reflect a conserved function for *usp*/RXR in eye dorsoventral patterning. That genetic mechanisms involved in eye development have been conserved between Drosophila and mouse was recently spectacularly demonstrated by the finding of a conserved master role of the *Pax6/eyeless* gene for eye determination (Halder et al., 1995).

Function of RXRβ

RXRβ null mutants are viable and morphologically normal, but RXRβ$^{-/-}$ males are sterile owing to abnormal spermatid maturation and release, leading to oligo-asthenoteratozoospermia (Kastner et al., 1996). The sperm abnormalities are preceded by the appearance of triglyceride droplets in Sertoli cells, which may be primarily affected since RXRβ is present in these cells, but not in germ cells. However, in contrast with RARα (see above), it is unlikely that these defects reflect a role of RXRβ in mediating the retinoid signal required for spermatogenesis, since testes from VAD mice do not accumulate lipids and degenerate much earlier. RXRβ may rather act as an heterodimeric partner for PPARβ, which is highly expressed in Sertoli cells (for reference see Kastner et al., 1996).

Compound Mutants Suggest That RXR/RAR Heterodimers Are the Functional Units In Vivo

Examination of mice deficient in RXRα and of various RXRα/RAR mutants has revealed a convergence between RXR- and RAR-dependent signaling pathways: the severity of several ocular abnormalities present in RXRα null mutants increases in a graded manner with the successive removal of the two alleles of either RARβ2 or RARγ (Kastner et al., 1994; Table 3); the inactivation of only one RXRα allele from a RARγ null genetic background can cause eye defects identical to those observed in RXRα null mutants (Table 3); new abnormalities, absent in RXRα null mutants, are generated in RXRα/RAR mutants (e.g., persistent truncus arteriosus in RXRα$^{-/-}$/RARα$^{-/-}$ mutants; see Table 3). Since all of these abnormalities are present in VAD fetuses, these synergistic effects of RXRα and RAR mutations strongly suggest that, at least in some instances, RAR/RXR heterodimers act as functional units transducing the RA signal in vivo. Thus, the analysis of RAR/RXR double mutants may lead to the identification of the RAR/RXR pair predominantly implicated in mediating the RA signal in a given process. For instance, the complete penetrance of the persistant truncus arteriosus defect in RXRα/RARα double null mutants, together with the absence of this defect in RXRα/RARβ2 double mutants

Table 3. Comparison of the Incidence and Severity of Malformations in RAR/RAR and RXRα/RAR Compound Mutants

	Mutations	
Defect	RXRα/RAR	RAR/RAR
Persistent truncus arteriosus	RXRα/RARα (+++)	α/β2 (+++)
	RXRα/RARα$^{+/-}$ (+)	α/γ (+++)
	RXRα/RARγ (+)	α/β2$^{+/-}$ (+)
	RXRα/RARγ$^{+/-}$ (−)	α$^{+/-}$/β2 (−)
	RXRα/RARβ2 (−)	β2/γ (−)
Malformations of the eye anterior segment	RXRα[a] (+)	α/γ (++)
	RXRα$^{+/-}$/RARγ (+)	β2/γ (++)
	RXRα/RARγ$^{+/-}$ (++)	α/β2 (−)
	RXRα/RARγ (+++)	
	RXRα$^{+/-}$/RARβ2 (−)	
	RXRα/RARβ2$^{+/-}$ (+)	
	RXRα/RARβ2 (++)	
	RXRα$^{+/-}$/RARα (−)	
	RXRα/RARα (+)	

These data show that the abnormalities that can be generated by either one of two compound RAR null mutations can be preferentially generated by a compound mutation of a specific RXR/RAR pair. Mutations are all homozygous null except where indicated. Minus indicates no defect. Plus to triple plus indicate either an increase of the incidence (persistent truncus arteriousus) or of the severity (malformations of the eye anterior segment) of the defect(s). For further details see Kastner et al. (1994).

[a] In all other cases, single RXRα or RAR null mutants did not exhibit any abnormality.

and its incomplete penetrance in RXRα/RARγ mutants, strongly implicates the RXRα/RARα pair is the main functional heterodimeric unit in the formation of the aorticopulmonary septum. That RARα plays a major role in this process can also be inferred from the comparison of RAR compound mutants (Table 3). Note that distinct RXR/RAR pairs may mediate the RA function in different NCC-dependent morphogenetic processes (aorticopulmonary septation and anterior eye segment development; Table 3).

A convergence of RXR and RAR function was also suggested by Roy et al. (1995), who have shown that a combination of limiting concentrations of RXR- and RAR-specific agonists can act synergistically to activate endogenous RA target genes in cultured EC cells. Thus, ligand activation of both RXR and RAR heterodimeric partners may be required under physiological conditions of low ligand levels to induce the expression of RA target genes.

RA Excess Studies and Physiological Function of RA

It has been widely accepted (see, for instance, the recent review by Means and Gudas, 1995) that the teratogenic effects resulting from administration of exogenous RA to embryos reflect a physiological role for endogenous RA in the corresponding developmental processes, e.g., as a morphogen for patterning of the limb anteroposterior axis. Recent genetic analyses of RAR and RXR functions cast some doubt on the physiological implications of RA excess studies. In the two instances in which an involvement of a given RAR or RXR in the mediation of a teratogenic event was demonstrated, the same receptor was clearly not required for the development of the corresponding structure during embryogenesis. This is the case for the RA excess-induced lumbosacral truncation that is mediated by RARγ (Lohnes et al., 1993) and the RA excess-induced limb truncations that do not occur in RXRα mutants (Sucov et al., 1995). Along the same lines, it has been shown that expression of a dominant negative RAR in Xenopus embryos could prevent RA-induced anterior truncations, although expression of the same mutant did not affect early axial patterning in the absence of addition of exogenous RA, which casts doubts on the presumed role of RA in this patterning (Smith et al., 1994).

Functional Redundancy: What Does It Mean?

In general, the defects generated by single knockouts of receptors belonging to a family (e.g., RARs, RXRs, TRs, PPARs, or NGFI-B/NURR1/NGFI-Bγ) were milder than expected. As this has been extensively studied for RARs and RXRs, the following discussion deals with these receptors.

The high degree of interspecies conservation of individual RAR isoforms throughout vertebrate evolution and the differential distribution of their transcripts, taken together with molecular biology studies on RARs and RXRs, have suggested that the basis for the pleiotropic effect of retinoids may reside in the control of different subsets of retinoid-responsive promoters by cell-specifically expressed dimeric combinations of different RARs and RXRs (see Chambon, 1994). It came therefore as a surprise that single knockouts of given RARs or RXRs had no apparent effect or had much milder effects than expected. In contrast, mutations affecting pairs of RARs yielded the full spectrum of expected defects, and in a number of cases, the same malformations occurred in different double mutants (Figures 1 and 2; Table 3), thus suggesting that the different receptors could be functionally redundant (interchangeable).

At one extreme, one could envisage that all RAR isoforms, as well as all RXR isoforms are mostly, if not fully, functionally redundant for the transcriptional control of all RA target genes. The only requirement would be to reach a certain threshold level of RAR and RXR in a given cell at a given time, which could be achieved through any combination of RAR and RXR isoforms. Multiple RAR and RXR genes with multiple promoters and alternative splicing would have evolved only to fulfil these purely quantitative spatiotemporal requirements. For instance, the observation that the Harderian gland is often unilaterally lacking in RARγ mutants (Lohnes et al., 1993), whereas it is always bilaterally missing in RARα1/γ double mutant, may indicate that RARγ and RARα1 are functionally redundant for Harderian gland ontogenesis and that, owing to stochastic variations of RARα1 levels, the critical quantitative threshold can be in some cases reached in the absence of RARγ (for further discussion on variations of penetrance and expressivity, see Chambon, 1994; Lohnes et al., 1994).

In fact, it would not be surprising that, within a given family that has evolved by duplications from an ancestral gene, the various receptor isoforms may still be functionally close enough to perform a number of common functions. However, studies with either RARγ or RARα null F9 EC cells have shown that the RA-induced differentiation of these cells and the RA inducibility of various target genes are differently affected by disruption of either RARα or RARγ gene, indicating that these receptors perform some specific functions in these cells (Boylan et al., 1993, 1995). Nevertheless, RARγ null cells could be rescued to some extent by overexpression of either RARα or RARβ, showing that RARα and RARβ can perform some of the functions normally exerted by RARγ, albeit with a lower efficiency (Taneja et al., 1995). These observations are in keeping with in vitro transfection studies that have shown that some promoters are preferentially activated by given receptors (Nagpal et al., 1993).

In any event, each isoform must possess at least one specific function so as to explain its striking sequence conservation across vertebrates, particularly of its N-terminal A region, which contains a transcriptional activation function (AF-1) (Nagpal et al., 1993). Along these lines, it is interesting that chimeric receptors containing the N-terminal region of RARα1 or RARγ1 (including the DBD) fused to the TR LBD displayed specific functions upon T_3 activation. In the context of regenerating newt limb blastema cells, only the RARα1–TR chimera could mediate an inhibition of cell proliferation, whereas the RARγ1–TR chimera specifically induced the expression of a differentiation marker (Schilthuis et al., 1993; Pecorino et al., 1994). Why then are some RAR isoform mutants apparently normal? An abnormal phenotype may not have been seen for

one of the following reasons: it corresponds to a discrete defect, which is not easily detected (e.g., a behavioral alteration); it corresponds to a function dispensable in the laboratory (e.g., resistance to harsh climatic conditions); it corresponds to a function that gives a low, but significant, viability advantage and that cannot be detected unless several thousand animals are examined (see Brookfield, 1992).

Thus, it appears notably from studies performed with F9 cells that the different receptor isoforms are not truly functionally equivalent, even though they can substitute for one another under certain conditions. How can we explain that double knockouts are often required to detect phenotypic abnormalities? The most likely explanation is that the absence of a given defect in a single mutant does not mean that the RA responsiveness of certain target genes is not impaired to some degree, but rather that the response mediated by the substituting receptor is still efficient enough. This is strongly supported by the observation that there is much less functional redundancy between RARs in an RXRα mutant genetic background (see Table 3). These observations can be readily accounted for by postulating that the actual functional units are RXR/RAR heterodimers. Knocking out both RXR and RAR partners of the specific RXR/RAR heterodimer that performs most efficiently a given function (e.g., RXRα and RARα in the case of aorticopulmonary septation) will result in the loss of that function, while knocking out either the RXR or the RAR partner only will allow functional substitution by another RXR or RAR, thus resulting in less efficient, but still functionally redundant, heterodimers. It will be only with the simultaneous mutation of RARα and either RARβ2 or RARγ that the efficiency of the remaining heterodimers will fall below a critical level. In other words, the conclusion that RARs exhibit a high degree of functional redundancy would be physiologically wrong because the functional units correspond to heterodimers, whose functional specificity can be evaluated only by the simultaneous mutation of both RAR and RXR partners. Much of the RAR functional redundancy seen in knockouts would not reflect lack of functional specificity, but rather potentialities linked to the complexity of the retinoid-transducing system, which are revealed under the "artefactual" conditions of particular gene knockouts. The assumption that heterodimers are the functional units transducing the retinoid signal also provides a satisfactory explanation to the otherwise puzzling observation that RAR compound mutants and RXR/RAR mutants very often exhibit the same defects.

Finally, functional redundancy is not the only possible explanation for the occurrence of a given abnormality in compound, but not in single mutants. More complex scenarios are not excluded in which multiple molecular defects generated by nonredundant receptors, acting on specific subsets of RA target genes, would have to be combined to generate visible abnormalities (for further discussion see Thomas, 1993; Mendelsohn et al., 1994a). Additional RAR/RXR heterodimer knockouts, as well as the analysis of the expression of the corresponding RA target genes, are necessary to determine whether the abnormalities generated by mutations of the receptors result from cell-autonomous or non-cell-autonomous developmental processes and ultimately to which extent any apparent phenotypic redundancy truly reflects a functional redundancy at the cellular and molecular levels (e.g., in the case of malformations of the eye anterior segments, which are observed in both RXRα/RARγ and RXRα/RARβ2 compound mutants; see Figure 1 and Table 3).

Conclusion and Perspective

The studies summarized and discussed here illustrate the indispensability of loss-of-function genetic studies through knockouts to determine the physiological role played by nuclear receptors. We have learned that the simple knockout of a given receptor may not result in the unambiguous characterization of its role in vivo, particularly when it belongs to a family comprising several members and/or when it is a partner within a functional heterodimer. It is also clear that many of the presently available mutations cannot reveal all of the functions of a given receptor within the whole animal or a given organ, because they result in either a lethal phenotype (e.g., RXRα and HNF4) or agenesis of that organ (e.g., gonads and adrenals in FTZ-F1 mutants). Obviously, the future resides in engineering mutants that are only partially deficient (e.g., in either one of the two activation function AF-1 and AF-2) and/or in creating spatiotemporally controlled knockouts in the animal. Clearly, these approaches, which may also help in identifying the human clinical symptoms associated with mutations in nuclear receptor genes, will blossom and will be limited only by the size of animal facilities! Finally, for many of the defects that have been observed to date, very little is known concerning the mechanisms operating at the organism, tissular, cellular, and molecular levels. In which cells is the primary defect(s)? What are the target genes affected? The present stage of description of the knockout-induced defects, which is mostly anatomical, should lead in the near future to new tissular, cellular, and molecular approaches making use of nuclear receptor mutants to explore the mechanisms underlying mammalian ontogenesis and homeostasis.

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